Research Article

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Synthesis, Characterization and Biological Activity Studies of Semicarbazone Ligand

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ABSTRACT

The ligand 1-(3-ethoxy-2-hydroxybenzilidene-4-phenylsemicarbazide), (L1) was synthesized and characterized with the help of Infra-red, Ultra-violet, ^{1}HNMR spectroscopy and X-ray crystallography. The spectral data also indicate that the ligand coordinates through the phenolic oxygen and the azomethine nitrogen atoms. Crystal data revealed that the semicarbazone act as bidentate ligand, making use of azomethine nitrogen atom and oxygen atom for co-ordination to the central metal atom. The Ligand L1 have been screened for their antibacterial activity against gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Escherichia coli and Pseudomonas aeruginosa.*The ligand L1 exhibited appreciable activity against gram positive bacteria *Staphylococcus aureus* and it is resistant to the fungal species such as *Candida albicans*, *Aspergillus niger and Macrophonia phaseolina.* The free ligand L1also shows higher IC_{50} values against MCF-7 cells indicating the less anticancer activity.

Keywords: Semicarbazone; X-ray crystallography; antibacterial and antitumour activity.

1. INTRODUCTION

Semicarbazone plays a key role in organic and biological chemistry. The semicarbazone linkage is an important functional group due to its extensive presence in natural products, pharmaceutical compounds and synthetic polymers. The most common traditional method for the synthesis of the semicarbazone derivative is that semicarbazide hydrochloride on treatment with pyruvic acid or acetone gives semicarbazone derivative. In recent years there has been considerable interest in the studies of semicarbazone due to their coordination modes when bound to metal. The anticancer activity of certain pyridoxal semicarbazone (3-hydroxyl-5-hydroxymethyl-2- methyl-4-pyridine-carboxaldehyde semicarbazide) has also been studied. The coordinating ability of semicarbazones to both transition and main group metallic cations is attributed to the extended delocalization of electron density over the semicarbazone skeleton, which is enhanced by substitution at N(4)-position.

Condensation of semicarbazides with aldehydes or ketones extends the electron delocalization along azomethine bond. 2-hydroxybenzaldehyde N(4)-substituted semicarbazones, as well as heterocyclic thiosemicarbazones, derived from the presence of several potential donor atoms, their flexibility, and their ability to coordinate in either neutral or deprotonated forms, have been the subject of extensive investigations, because of their ability to strongly coordinate metal ions as bidentate ligands and their wide spectrum of biological applications (Leovac *et al.* 2014). Due to their good complexing properties, biological activity, and analytical application, semi-/thiosemi /isothiosemicarbazides and their Schiff bases of different denticity, as well as their metal complexes, have been the subject of many studies. The most numerous among them are the complexes with bi/tridentate salicylaldehyde semi/thiosemi-/isothiosemicarbazones (Ahmed *et al.* 2015).

Semicarbazones and thiosemicarbazone of salicylaldehyde (Kalaivani *et al.* 2012; Hossain *et al.* 2017) and its derivatives are a class of versatile ON/ONS donors capable of stabilizing both higher and lower oxidation states of transition metal ions (Pahontu *et al.* 2015; Selvaganapathy *et al.* 2016). Although capable of deprotonation at both the phenol and thioamide functions to give a dianionic ligand, they can also act as mono anionic chelating ligands, coordinating to a metal centre phenolic oxygen, the thione sulfur and azomethine nitrogen (HAQUE *et al.* 2015). The dianionic form of the ligand is favored at higher through the deprotonated pH, whereas the monoanionic form is promoted at low pH. However, the coordination chemistry of substituted or unsubstituted semicarbazones and thiosemicarbazones of salicylaldehyde is quite unexplored with a few previous reports (Kumar *et al.* 2015). This prompted our study into the synthesis and characterization of substituted semicarbazones using aromatic aldehydes and its metal

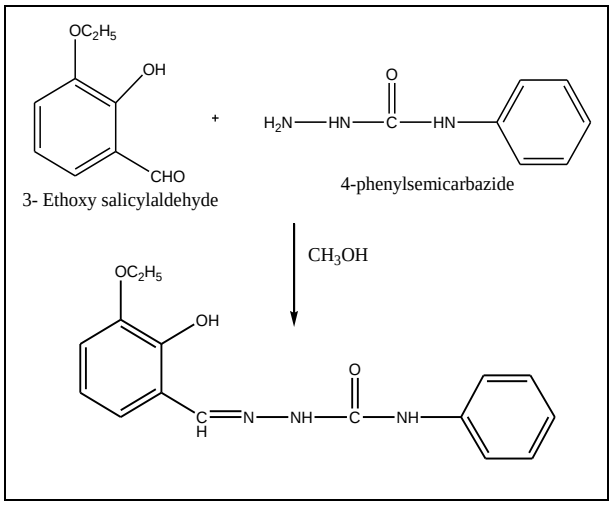
complexes. Here we have synthesized the new semicarbazone ligand using 3-Ethoxy salicylaldehyde and 4-Phenylsemicarbazide.

This paper describes the synthesis of semicarbazone ligand (L1), and various physico chemical methods employed for the characterization of the newly synthesized semicarbazone ligand and its biological applications.

2. EXPERIMENTAL

2.1 Synthesis of Semicarbazone Ligand L1

About 499 mg (3.00 mmol) of 3-Ethoxy salicylaldehyde in 5 ml methanol was treated with 454mg (3.00 mmol) of 4-phenylsemicarbazide in 5ml methanol and stirred for 2 hours. Then it is refluxed for 3 hours in a round bottomed flask. The solution was chilled (overnight) and fine colorless needles of the compound separated out. The solution was filtered, washed well with cold methanol. The compound was recrystallized from methanol and dried in vacuo over P_40_{10} (Scheme 1).



Scheme 1: Synthesis of Semicarbazone ligand L1

3. RESULTS AND DISCUSSION

The newly synthesized semicarbazone ligand is characterized by using single crystal X-ray diffraction, IR spectra, electronic spectra and ^{1}H NMR spectra.

3.1 X-Ray Crystallography

The structure of semicarbazone Ligand L1 with thermal ellipsoids at 50% probability was shown (Fig.1). The present compound crystallizes in the monoclinic system with C2/C space group (Table 1). There is only one molecule in the asymmetric unit (Fig. 2). The bond lengths and bond angles of the molecules are presented in the table 2. In the present structure the atoms N_1 and O_3 are in trans conformation with respect to N2 -C_8 bond (Fig.3) by the torsion angle O_3-C_8-N_2-N_1 =176.50 as in the structure of salicylaldehyde N(4)-Phenylthiosemicarbazone (Seena *et al.* 2008) and 2-hydroxyacetophenone N(4)-cyclohexythiosemicarbazone (Strehler *et al.* 2015). It is also supported by the bond lengths C_8-O_3 =1.22 and C_8-N_2 =1.3633. C_8-O_3 bond length is double bond length. But N_1-N_2 =1.3699 and C_8-N_2 =1.3633 are intermediate between single and double bonds as in the structure of salicylaldehyde N(4)– Phenylthiosemicarbazone (Seena *et al.* 2008). O_3 atom is trans to the hydrozinic N_1 atom as in the structures of salicylaldehyde semicarbazone (Enyedy *et al.* 2014), Benzaldehyde semicarbazone salicylaldehyde thio semicarbazone (Pahontu *et al.* 2013). The mean deviation calculations show that the semicarbazone moiety C_7-N_1-N_2-C_8-O_3-N_3 is nearly planar with a deviation 0.293 at C_7 and -0.0393 at N_1. Ring puckering and least square plane analysis show that the rings are in the same plane and these rings are not deviated from the central semicarbazone moiety.

3.2 Hydrogen Bonding

The present structure is stabilized by both inter and intra molecular hydrogen bonds (Fig.1). The intra molecular hydrogen bonds are C_{10}-H_{10}….O_3& O_1-H_{10}….O_2.The lists of hydrogen bonds present in the structure are given in table 3. The imine N atom is not involved in the hydrogen bonding with O_1 of phenyl ring since the N_1 atom are in trans conformation with O_1.The hydrogen bonds N_2-H_2N…O_1 (-x+1,-y+1,-z) and O_1-H_1…O_3 (-X+1,-Y+1,-Z) formed a closed loop R^2_2 (5) andN_2-H_2N…O_1 hydrogen bond itself formed a R^2_2 (14) hydrogen bonding motif.

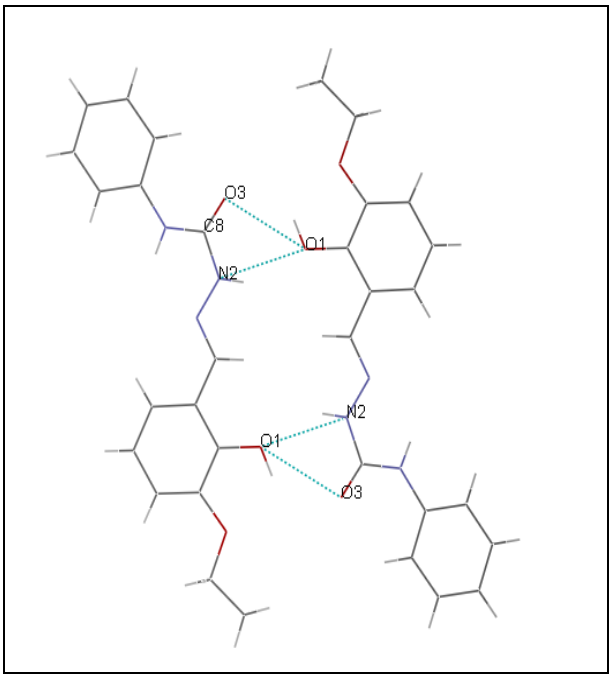


Fig. 1: Structure of semicarbazone Ligand L1

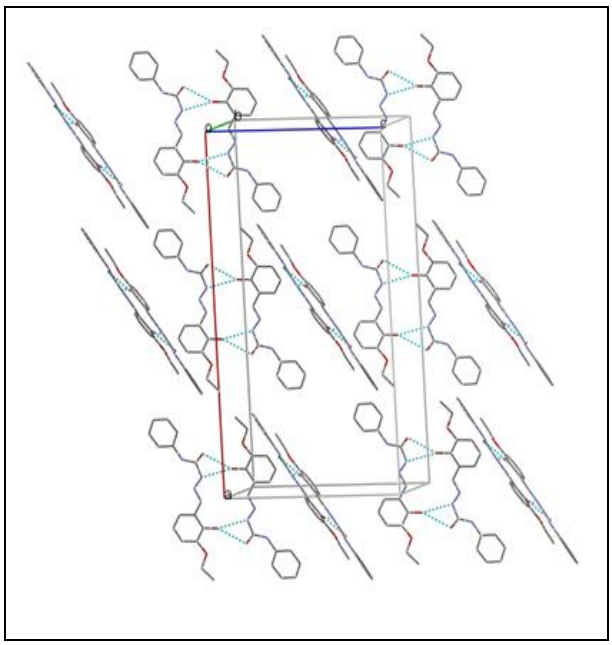


Fig. 2: Unit cell packing diagram of semicarbazone Ligand 1

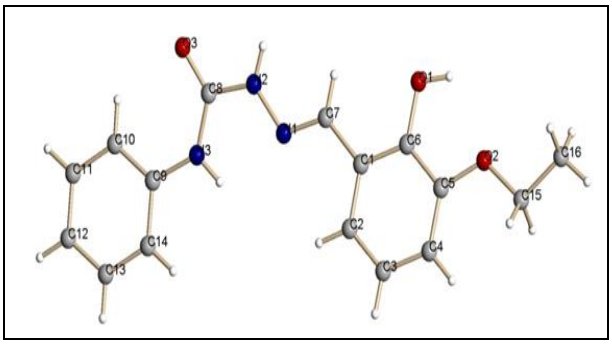


Fig. 3: Monomeric structure of the semicarbazone ligand

Table 1. Crystal data and structure refinement for thiosemicarbazone ligand 1

Crystal data	Ligand 1
Empirical Formula	$C_{16}H_{17}N_3O3$
Formula weight	299.33
Wavelength Aº	0.71073
Crystal system	monoclinic
Space group	C2/C
A, b, c [Angstrom]	30.131(2), 5.5670(4), 18.2336(15)
Alpha, beta, gamma [deg]	90, 92.660(2), 90
V [Ang ** 3]	3055.2(4)
Z Value	8
D(calc) [g/cm** 3]	1.301
F(000)	1264
Absorption coefficient mm^-1	0.092
Temperature K	294(2)
Theta Min-Max [Deg]	2.24 to 25.00
Completeness to theta	25.00 99.9%
Refinement method	Full matrix least-square on F^2
Final R indices [I>2sigma(I)]	R1 = 0.0349, wR2 = 0.0970
R indices (all data)	R1=0.0465, wR2=0.1104
Largest diff. Peak and hole e.A^-3	0.138 and -0.104

Table 2. Selected Bond lengths [A] and angles [deg] for Ligand1

Atoms	Bond length
C(5)-O(2)	1.3703 (17)
C(6)-O(1)	1.3635(16)
C(7)-N(1)	1.2762(16)
C(8)-O(3)	1.2264(17)
C(8)-N(3)	1.3533(18)
C(8)-N(2)	1.3632(18)
C(9)-N(3)	1.4105(17)
C(15)-O(2)	1.4246(18)
N(1)-N(2)	1.3701(17)
N(2)-H(2N)	0.840(18)
N(3)-H(3N)	0.860(17)
O(1)-H(1)	0.88(2)

Table 3. Selected Bond lengths [A] and angles [deg] for Ligand1

Atoms	Bond angle
O(2)-C(5)-C(4)	126.64(13)
O(2)-C(5)-C(6)	113.35(12)
O(1)-C(6)-C(1)	119.26(12)
O(1)-C(6)-C(5)	120.01(12)
N(1)-C(7)-C(1)	122.47(12)
N(1)-C(7)-H(7)	118.8
O(3)-C(8)-N(3)	125.82(13)
O(3)-C(8)-N(2)	119.87(13)
N(3)-C(8)-N(2)	114.31(13)
C(7)-N(1)-N(2)	115.78(12)
C(8)-N(2)-N(1)	122.75(12)
C(8)-N(3)-C(9)	128.36(12)
C(6)-O(1)-H(1)	109.7(13)
C(5)-O(2)-C(15)	118.79(12)

3.3 IR Spectra

IR spectra of L1 show bands at 3315 and 3390 cm^{-1}region due to intermolecular hydrogen bonded phenolic - OH groups (Table 4). It also suggests that the ligand exist in enol form in the solid state [18] present in the molecule. The azomethine stretching vibrations, C=N, characteristics of a Schiff and the coordination mode of ligand is supported by the disappearance of these bands in the spectra of complexes (Shimazaki *et al.* 2011; Abu-Khadra *et al.* 2016). The ligand have bands in the range of 2546 - 2900 cm^{-1} due to - NH groups base, are observed at ~ 1681 cm^{-1} (Sumrra *et al.* 2014; Shirode, P. R., Yeole *et al.* 2014). The carbonyl group shows stretching and bending vibrations at ~ 1319 and887 cm^{-1} while additional bands in the broad region of 1543-748 cm^{-1} are due to vibrations involving interactions between C=O stretching and C—N stretching attached to a nitrogen atom (Mandal *et al.* 2016). Medium bands observed in the range1064-1149cm^{-1} are assigned to hydrazinic N—N bonds (Mangamamba *et al.* 2014). The 1681-1450 cm^{-1}region of the spectra is complicated by the presence of ring breathing vibrations of the phenyl rings (Fig.4).

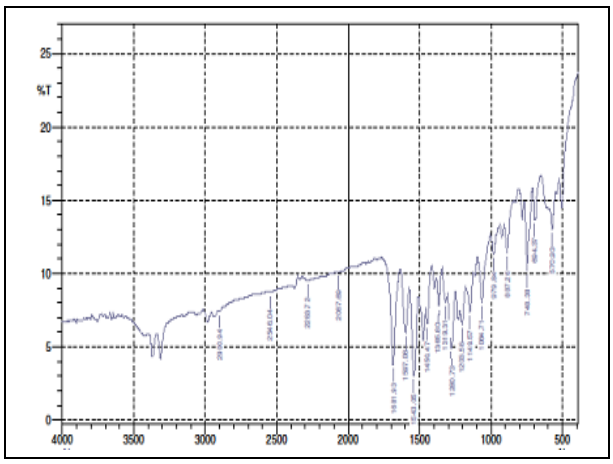


Fig.4. IR spectra of the newly synthesized ligand (L1)

Table 4. IR spectral data (cm^{-1}) and UV spectral data (nm) of free ligand (L1)

Ligand 1	Compound
1543	$\upsilon_{(CH=N)}$
1280	$\upsilon_{(C-O)}$
3390	$\upsilon_{(N-H)}$
748	$\upsilon_{(C=S)}$
3315	$\upsilon_{(O-H)}$
---	$\upsilon_{(M-N)}$
---	$\upsilon_{(M-O)}$
291 - 332	λ_{max} (nm)

3.4 Electronic Spectra

In contrast to the infrared spectrum, the electronic spectrum is not used primarily for the identification of individual functional groups, but rather to show the relationship between functional groups, chiefly conjugation. The electronic spectral data of the ligand in DMF solution are presented in Table 5. The π—>π* transitions of the phenyl ring are observed in the 263-291 nm region (Fig.5).The n→π* transitions of the imine function of the semicarbazone moiety are observed in the region of 301-332nm (Goel *et al.* 2013).

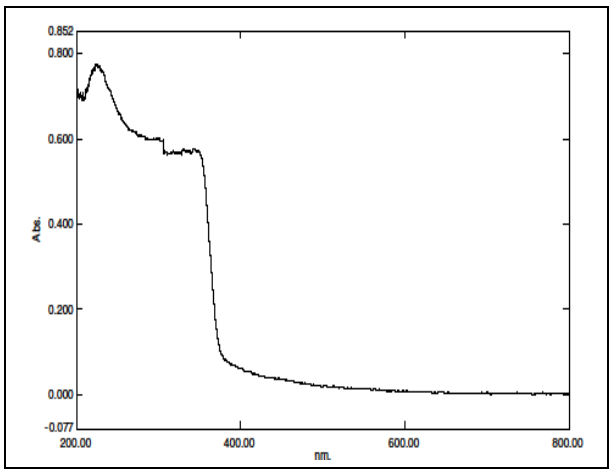


Fig. 5: Electronic spectrum of the ligand (L1)

3.5 ^{1}h NMR Spectra

Proton Magnetic Resonance spectroscopy is a helpful tool for the preparation of organic compounds in conjugation with other spectrometric information. The ^{1}H NMR spectra of the ligand recorded in $CDCl_3$ (Table 5). The ligand do not show any peak attributable to -SH proton but they show peaks assignable to the secondary N-H protons. In the spectra of the ligand, sharp singlets at 7.64 and 8.29ppm is due to CH proton (Fig.6). Absence of any coupling interactions by ^{2}NH due to the unavailability of protons on neighboring atoms render singlet peak for the imine proton at 8.87ppm. The $^{a}CH_2$ protons adjacent to the ring nitrogen produces a triplet at 4.02ppm due to coupling with nearby $^{b}CH_2$ protons. The ^{b}CH2 protons due to coupling with $^{a}CH_2$ and $^{c}CH_2$ protons resonate as the multiplet observed at 1.38 ppm. The OCH3 protons appear as a singlet at 3.37 ppm. The spectra of the ligand (L1) also show sharp singlets, which integrates as one hydrogen at ~ 10.67 ppm is assigned to the proton attached to the oxygen atom. The downfield shift of this proton is assigned to its intra and intermolecular hydrogen-bonding interactions. The hydrogen bonding decreases the electron density around the proton, and thus moves the proton absorption to a lower field . Absence of any coupling interactions by ^{2}NH due to the unavailability of protons on neighboring atoms render singlet peak for the imine proton at 8.87ppm. The presence of electron withdrawing azomethine group near to the ^{7}CH proton leads to its resonance as a singlet at 7.64ppm. Aromatic protons ^{4}CH, ^{6}CH, ^{3}CH, ^{5}CH appear as a multiplet in the range of 6.76-7.31 ppm [30]. NMR assignments are in agreement with values already reported.

3.6 Anion Sensing Analysis

3.6.1 Colorimetric Analysis

In order to deduce the anion sensing ability of the sensor (L1) with halide anions (F^- , Cl^-, Br-and I^-) experiments were carried out in different solvents namely $CHCl_3$, CH_3CN and DMSO. The change in optical and optoelectronic properties was monitored by visual (naked-eye). First, the halide anions (F^-, Cl^-, $Br^-$and I^-) were added as tetrabutylammoniumsalts (0.2 equiv) to solutions of the sensor (L1) in chloroform/acetonitrile/DMSO. In the naked eye experiments, the sensor (L1) (5×10^{-5} M) showed dramatic color change from colorless to pale yellow, in the presence of tetrabutylammoniumfluoride (0.2 equiv) (Fig.7). The reason for this color change was probably due to the formation of hydrogen bond interactions between the two NH groups of the ligand and fluoride ions. The receptor was found to be in sensitive even on addition of large excess of Cl^-, $Br^-$and I^- (up to 100 equiv). No color change ($CHCl_3$ and DMSO medium) was observed in the presence of chloride, bromide and iodide ions as similar to acetonitrile medium. In the receptors, it has been speculated to be a hydrogen bond-donor and suitable to interact with the anions, has been appended in between substituted-aldehyde and amine units.

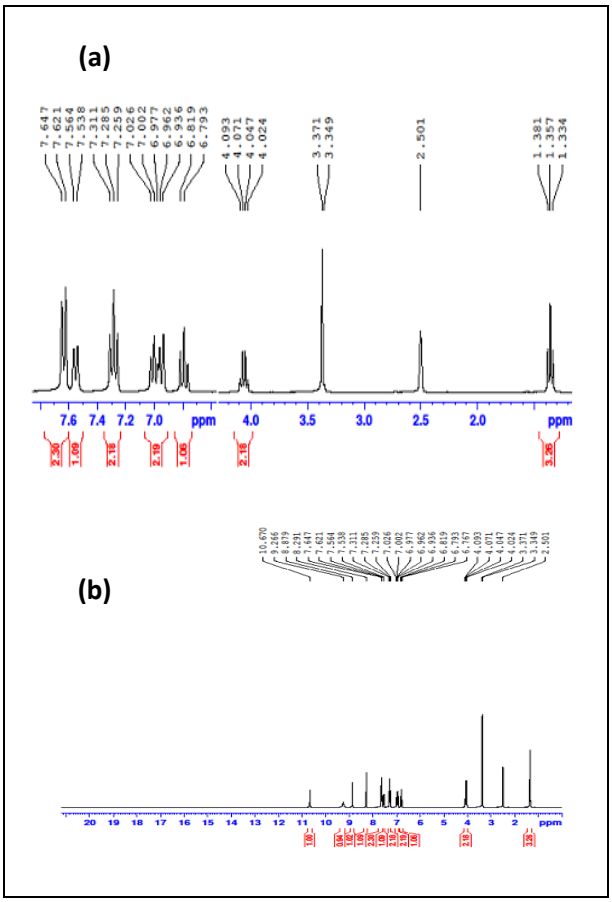


Fig. 6: (a) ^{1}H NMR spectrum of the ligand in $CDCl_3$- d_6 (b) Expanded form of L1

The covalently linked 4-phenylthiosemicarbazone moiety intends to act as a chromospheres unit (Li *et al.* 2012). The experimental results indicate that this receptor is highly selective compared with similar motifs and

sensitive to recognize fluoride anion in dry CH_3CN and the processes of sensing can be obviously seen through the visible color changes for naked-eye recognition.

Table 5. ^{1}H NMR spectral data of the L1 (in ppm)

Ligand 1	Compound
10.67	υ (O-H)
8.87	υ (CH=N)
6.76 – 7.31	Aromatic
3.34 – 3.37	Aliphatic

Fig. 7: Color changes of sensor (L=L1) (5.0×10$^-$5 M) in CH_3CN before and after addition of 0.2 equiv of representative anions (from left to right:L,L+F$^-$, L+Br$^-$, L+Cl$^-$, L+I$^-$, L+Aco$^-$, L+ CN$^-$)

3.6.2 UV Kinetics

The interaction of sensor 1 with the fluoride anion was investigated through spectrophotometric titrations, by adding a standard solution of tetrabutylammonium salt of the anion in acetonitrile of the free receptor and for the titration of sensor 1 with F^-, was shown in Fig.8. The titrations were carried out in CH_3CN at 5.0 × 10^{-5}M of L1 upon the addition of incremental amounts of 0.02 ml (5.0× 10^-4 M) of tetrabutylammonium fluoride and the changes in UV–Vis spectra are observed. In the absence of anions, the UV–Vis absorption spectrum of 1 was characterized by the presence of four absorption maxima. The first two bands(210–280 nm) could be assigned to excitation of the p-electrons of the aromatic system, the third band 315 nm is due to the transition between the p-orbital localized on the azomethine group(CH=N). The band in the region of 405 nm may occur due to intramolecular charge-transfer transitions within the whole structure of the Schiff base. Upon addition of F^-, a dramatic change in the spectra was observed. The peaks at 315 and 405 nm of the free receptor gradually decreases and a new absorption peak appears at 460 nm, as the concentration of F^- increases due to the formation of a complex with the receptor. A bathochromic (red) shift of the absorption maxima (460 nm) in the visible region of the spectra was observed on complexation of receptors with F^-. This is presumably due to charge-transfer after the interaction between the proton of the amine group and the acceptor (anion) groups (El-Asmy *et al.* 2009; Ghosh *et al.* 2011).

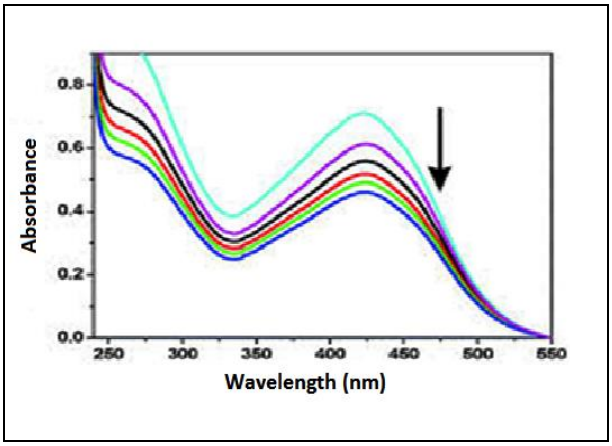


Fig. 8: The changes in the absorption spectra of L1 (5 × 10$^-$5M) upon addition of F$^-$(0 -100 equiv) in CH_3CN, demonstrating that the anion can interact with semicarbazone moiety, giving rise to large changes in the ground state properties of the sensor.

3.7 Biological Studies

3.7.1 Antibacterial Activity

The newly synthesized Ligand was tested for their antibacterial activity by measuring the inhibition area on agar plates (diffusimetric method) with the standard drug Amikacin were screened separately for their antibacterial activity against the gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa.* The results of antibacterial activity of semicarbazone Ligand was listed in table 6 as estimated by zone of inhibition (mm).The results show that free semicarbazone ligand (L1) have effective activity against Gram positive bacteria *Staphylococcus aureus and shows resistivity against* Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Fig.9)

3.7.2 Antifungal Activity

From the data table 6. indicate that the ligand **(**L1**)** have a less degree of antifungal activity against, *Candida albicans*, *Aspergillus niger* and *Macrophonia phaseolina* at 2 mg/ ml concentration. The effect is susceptible to the concentration of the compound used for inhibition. The antifungal experimental results of the compounds were compared with the standard antifungal drug Ketokonazole at the same concentration. From the observed data (Table 6) it shows that the antifungal activity of the ligand (L1**)** shows resistant against the fungal species (Fig.9a).

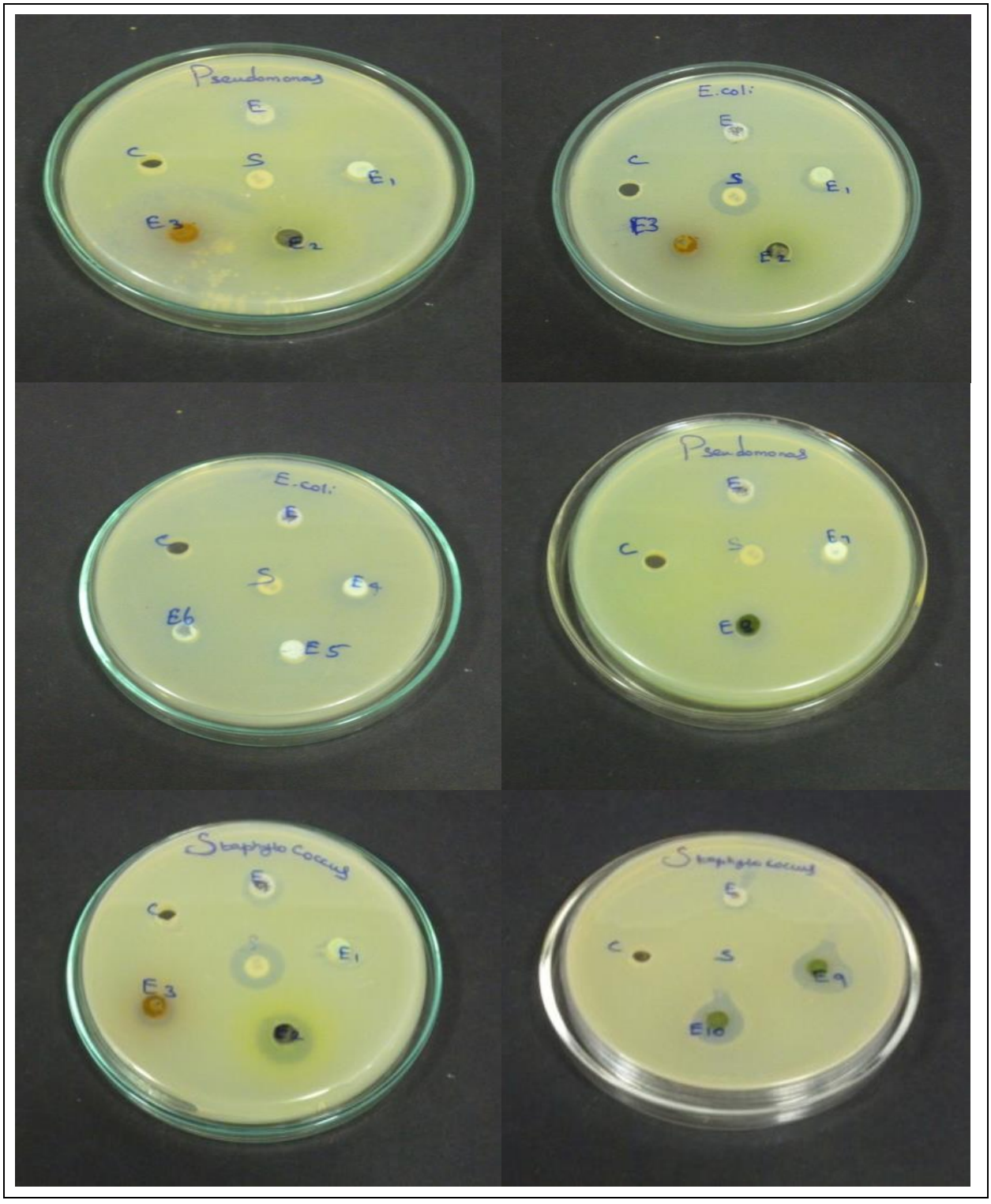


Fig. 9: Images of zone of inhibition in ligand L1 against bacteria

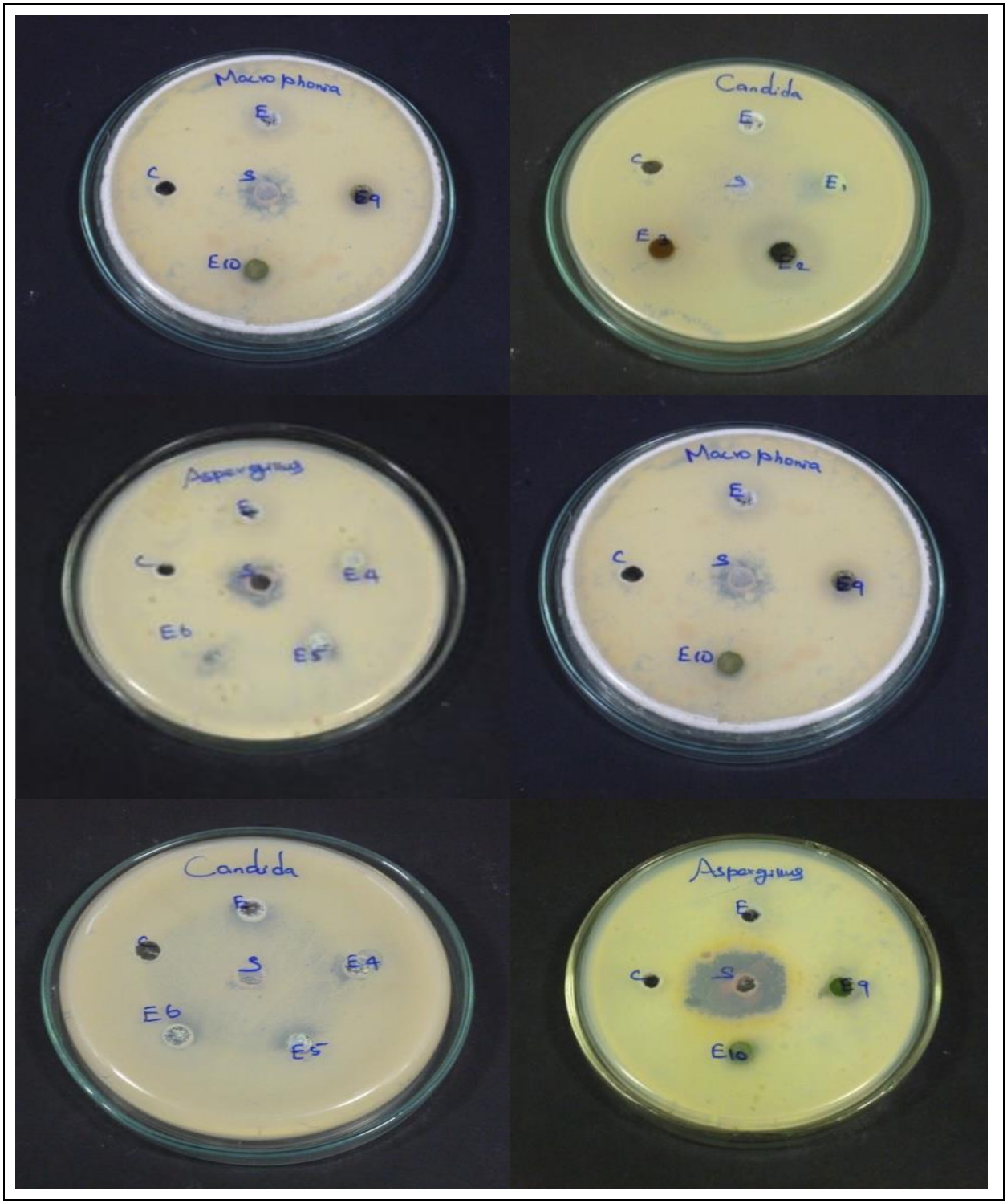


Fig. 9: (a) Images of Zone Inhibition in ligand (L1) against fungi

Table 6. Antimicrobial activity of the newly synthesized Semicarbazone ligand (L1)

Sample	*E.coli*	*Staphylococcus aureus*	*Pseudomonasauroginosa*	*Candidaalbicans*	*Aspergillusniger*	*Macrophonia phaseolina*
Ligand 1	R	4	R	R	R	R
STANDARD	17	18	17	21	17	15

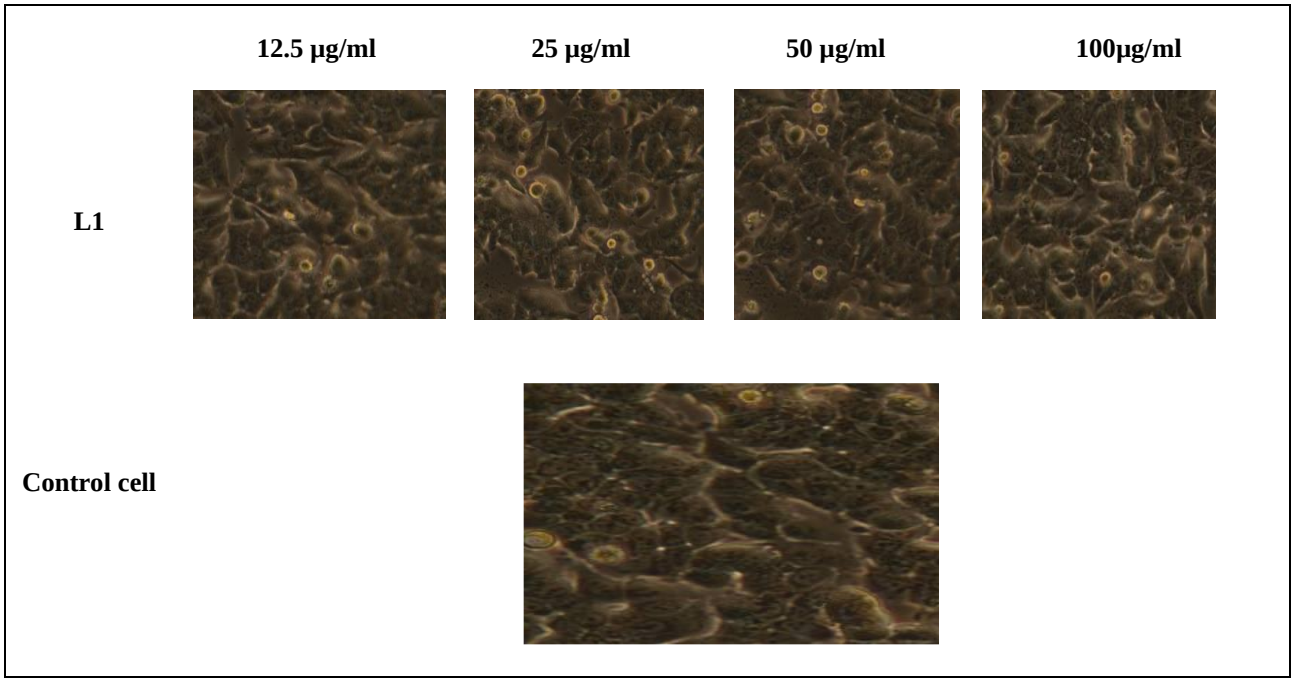


Fig. 9.1: Morphological changes in MCF-7 cells incubated for 24 h compound of ligand.

The cells are observed under phase contrast microscope × 100.

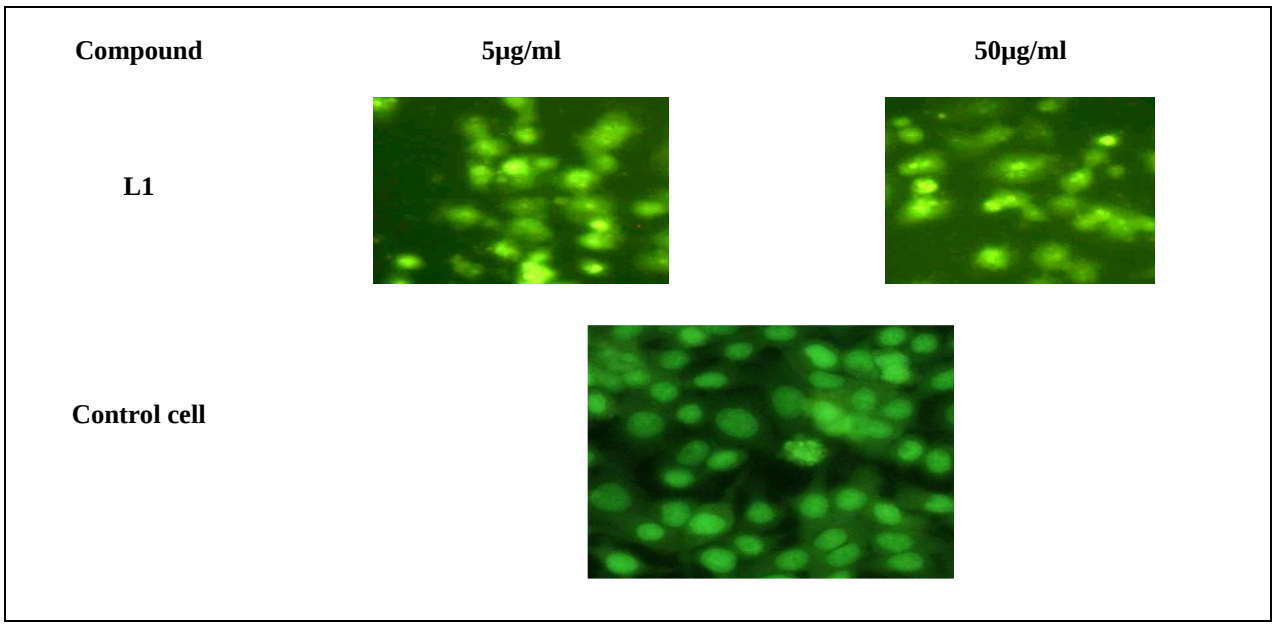


Fig. 9.2: Fluorescent Staining Studies of the synthesized ligand (L1) and control cell.

Table 7. IC_{50} values of the ligand L1

S. No	Sample	IC_{50} values
1.	Ligand 1	112
2.	Cyclophosphamide	100

3.8 Invitro Cytotoxic Activity

The *in vitro* cytotoxic activities of the synthesized Schiff base ligand (L1) was studied on human breast cancer cell lines (MCF-7) by applying the MTT colorimetric assay (Table 7). IC_{50} values (compound concentration that produces50% of cell death) were calculated for the free ligand. Cyclophosphamide is chosen as a reference compound. It is worth noting that the free ligand shows higher IC_{50} values, indicating that the antitumor activity of the free ligand shows lesser activity against human breast cancer cell lines (MCF-7).

3.9 Fluorescent Staining Studies

Apoptosis (programmed cell death) is a normal component of the development and maintenance of health of multicellular organisms. Cells die in response to a variety of external and physiological stimuli, and during apoptosis, they do so in a controlled and regulated fashion. This makes apoptosis distinct from the other form of cell death, namely, necrosis in which uncontrolled (accidental) cell death leads to lysis of cells, to inflammatory responses and potentially, to serious health problems. Moreover, apoptosis by contrast, is a process in which cells play an active role in their own death (cell suicide). Most tumor cells retain their sensitivity to some apoptotic stimuli from chemotherapeutic agents, and in this context, the apoptosis-inducing ability of drugs seems to be a primary factor in determining their efficacy.

In the present study, the characteristic morphological changes induced by the ligand L1 have been evaluated by adopting fluorescent microscopic analysis of Acridine orange/EthBr (AO/EB)-stained cells. Upon treatment of the cells with IC_{50} concentration of L1 at different incubation time 24 hrs, morphological changes such as chromatin fragmentation, bi- and/or multinucleation, cytoplasmic vacuolation, nuclear swelling, cytoplasmic blebbing, and late apoptosis indication of dot like chromatin condensation [42] have been observed by adopting AO/EB staining. All the morphological changes observed for the ligand L1 suggest that the cells are committed to death in such a way that both apoptotic and necrotic cells increase in number in a time-dependent manner (Fig.9.2).

CONCLUSION

1- (3- ethoxy-2-hydroxy benzilidene -4-phenylsemicarbazide) the ligand L1have been prepared and characterized by spectral techniques and X- ray crystallography. The spectral data also indicate that the ligand coordinates through the phenolic oxygen and the azomethine nitrogen atoms. Crystal data revealed that the semicarbazone act as bidentate ligand, making use of the azomethine nitrogen atom and oxygen atom for co-ordination to the central metal atom. Further in the anion sensing analysis the color change was seen with naked eye is probably due to the formation of hydrogen bond interactions between the two NH groups of the ligand and fluoride ions. The antibacterial activity results show that free semicarbazone ligand (L1) have effective activity against gram positive bacteria *Staphylococcus aureus* and it is resistant to the fungal species. The free ligand L1 also shows higher IC_{50} values against MCF-7 cells indicating the less anticancer activity.

SUPPLEMENTARY DATA

CCDC 923703 contains the supplementary crystallographic data for 3-(ethoxy-2-hydroxybenzylidene)-4-phenylsemicarbazide. . These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

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